Brief Definitive Report

INVASION OF MOUSE ERYTHROCYTES BY THE HUMAN MALARIA PARASITE, *PLASMODIUM FALCIPARUM*

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Malaria merozoites initiate erythrocyte invasion through interactions between merozoite surface receptors and complementary erythrocyte surface ligands (1). *Plasmodium falciparum* merozoites require erythrocyte sialic acid for optimal invasion (1, 2). Some strains (e.g., the Camp strain) have an almost absolute requirement for sialic acid; others do not (2; Hadley, T. J., F. W. Klotz, G. Pasvol, J. D. Haynes, M. H. McGinniss, Y. Okubo, and L. H. Miller, unpublished data) (i.e., they invade erythrocytes depleted of sialic acid by neuraminidase). A large proportion of the erythrocyte surface sialic acid is linked to glycophorins. It was believed that peptides of glycophorins A and B contribute to the sialic acid–dependent ligand for invasion. However, *P. falciparum* invades M^kM^k human erythrocytes that are deficient in glycophorins A and B (Hadley, T. J., et al., unpublished data). Furthermore, the sialic acid on M^kM^k cells required by Camp parasites is not carried on the glycophorins (Hadley, T. J., et al., unpublished data). Since mouse erythrocytes contain *N*-acetyl neuraminic acid (3), the form of sialic acid found on human erythrocytes, we tested mouse erythrocytes for susceptibility to invasion. This report documents invasion of mouse erythrocytes by the human malarial parasite *P. falciparum*.

Materials and Methods

Erythrocytes and Parasite Culture. Blood was collected in an anticoagulant solution (acid citrate dextrose, ACD) from a rhesus monkey, 2-d-old chickens, B10.BR mice, other mouse strains, Sprague-Dawley rats, and O^+ human donors. Cells were washed free of plasma proteins and stored refrigerated in RPMI 1640 containing 10% heat-inactivated FCS (Gibco Laboratories, Grand Island, NY). Before culture, erythrocytes were washed to remove FCS. Two cloned strains of *P. falciparum* were studied: the Malayan Camp strain (cloned at the Walter Reed Army Institute of Research, Washington, DC) and the Brazilian 7G8 strain (4). *P. falciparum* parasites were cultured as previously described (5, 6).

Invasion Assay. The method of purification of schizont-infected erythrocytes (7) and the erythrocyte invasion assay (8) were as previously described. In brief, mature schizont-infected erythrocytes and uninfected target cells were mixed at a ratio of schizonts to target cells of 1:5 and 1:10 for Camp and 7G8 parasites, respectively, and were cultured in heat-inactivated human O^+ serum preabsorbed against the panel of target erythrocytes. Merozoites are released from schizont-infected erythrocytes during culture and invade

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TABLE I

A Representative Experiment Illustrating Invasion of Various Species' Erythrocytes by Two Strains of P. falciparum (Camp and 7G8)

Target erythrocyte	Camp*	7G8*
Human	15.5	36.7
Mouse	4.7	9.2
Rhesus	0.1	0.2
Chicken	0.1‡	0.1‡

* Data are expressed as the mean percentage of ring-infected erythrocytes for triplicate samples.
‡ No ring-infected chicken erythrocytes. All infected cells were nonnucleated.

target erythrocytes. The proportion of newly invaded target cells that contain ring stage parasites is determined from Giemsa-stained blood films obtained 20–24 h after target erythrocytes were mixed with schizont-infected erythrocytes.

Immunofluorescence Studies. 24 h after initiation of culture with target cells and schizont-infected erythrocytes parasite cultures were washed free of human serum proteins and resuspended in heat-inactivated FCS (50%, vol/vol). Cells were mounted on glass slides as thin films and air dried. Cells were fixed in absolute methanol at −20°C for 20 min and rehydrated with PBS. Blood films were reacted in the first step with diluted rat anti–mouse erythrocyte serum (a gift from Drs. Paul Brindley and Ed Pearce, NIH) and rabbit antiserum that reacts with the 155 kD *P. falciparum* protein associated with ring-infected human erythrocytes (9) (a gift from Dr. Alan Saul, NIH). Immunofluorescent reagents used in the second step were fluorescein-conjugated goat anti–rat IgG and rhodamine-conjugated goat anti–rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL). All smears were incubated with antibodies in a humidified chamber and mounted in 90% glycerol in PBS containing 1 mg/ml *p*-phenylene diamine (10) for photography.

Ultrastructural Studies. To deplete human erythrocytes from parasite cultures containing mouse target erythrocytes, the cells were washed free of human serum and resuspended in RPMI 1640 containing 2% (wt/vol) BSA. The cell suspension (1 ml) was mixed with 100 μl of rabbit antiserum against human erythrocytes preabsorbed with mouse erythrocytes (a gift from Dr. Richard Carter, NIH) and incubated for 20 min at 37°C. Agglutinated human cells were allowed to settle and the unagglutinated mouse cells were washed. The process was repeated twice and the final cell suspension was washed free of rabbit serum, resuspended in RPMI/2% BSA, and adsorbed to a column of protein A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) to remove any remaining IgG-coated human erythrocytes. The effluent cells contained 5% ring-infected erythrocytes as determined by a Giemsa-stained blood film and <0.01% human erythrocytes as determined by immunofluorescence. For ultrastructural studies, cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and postfixed in 1% OsO_4 in cacodylate buffer.

Results

In the invasion assay, purified schizont-infected erythrocytes from two strains of *P. falciparum* were cultured separately with human, rhesus, chicken, mouse, and rat target erythrocytes. Chicken erythrocytes, which are nucleated, were completely refractory to invasion by *P. falciparum* (Table I). The few ring-infected erythrocytes in the culture were nonnucleated, indicating that a small number of normal human erythrocytes were introduced with the schizont-infected erythrocytes for the invasion assay. The low invasion rate into rhesus erythrocytes (Table I) probably also indicates that these cells are refractory to invasion as had been reported previously (11).

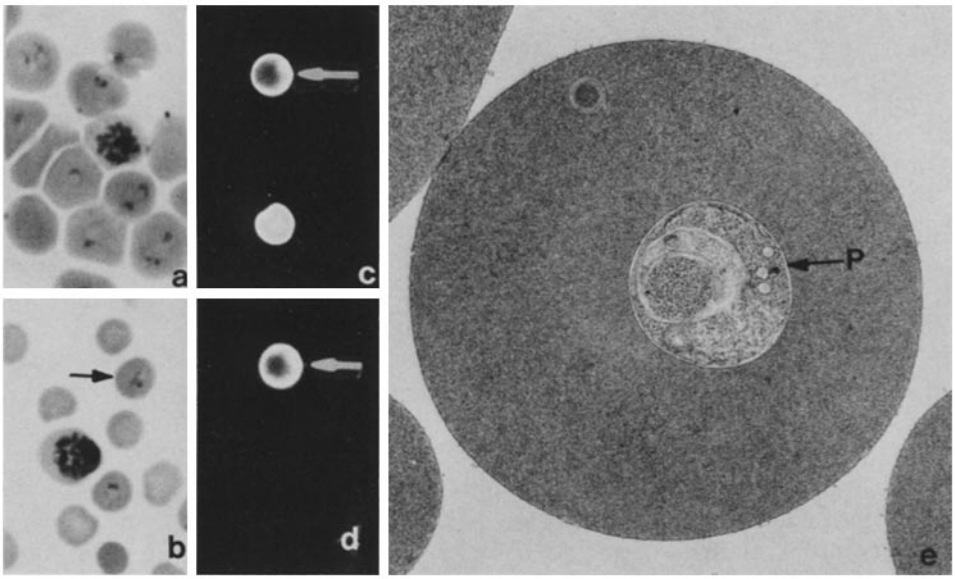

FIGURE 1. Bright field microscopy of parasitized human (*a*) and mouse (*b*) erythrocytes stained with giemsa. An arrow identifies an infected mouse erythrocyte. Immunofluorescence microscopy of erythrocytes reactive with anti-mouse erythrocyte serum (*c*) and with antibodies against the *P. falciparum* 155-kD protein (*d*). (*e*) Electron microscopy of a parasitized (*P*) mouse erythrocyte. (*a* and *b*) × 1,000; (*c* and *d*) × 1,100; (*e*) × 19,000.

In contrast, mouse erythrocytes contained a high proportion of ring-infected erythrocytes (Table I). The invasion rates, expressed as percentage of invasion with human erythrocytes, in four experiments with Camp parasites and in five experiments with 7G8 parasites were 34 ± 10 and 27 ± 9 (mean ± SD), respectively. Whereas, when these experiments were performed in a congenic mouse strain (B10.BR), *P. falciparum* parasites also invaded erythrocytes from a variety of other mouse strains including BALB/c, DBA/2, CBA/N, C57Bl/6, and outbred Swiss mice. In two experiments, Camp parasites invaded rat erythrocytes (8 and 17% of human control); 7G8 did not invade rat erythrocytes, that is, the invasion rate was at the low level of the chicken and rhesus erythrocyte background.

To prove that *P. falciparum* parasites had invaded mouse erythrocytes and were not just superimposed on Giemsa-stained blood films, we studied the ultrastructure of ring-infected mouse erythrocytes depleted of human cells. As seen in Fig. 1 *e*, parasites were indeed within mouse erythrocytes.

To determine if the invasion process was similar in mouse and human erythrocytes, we observed the distribution of a 155-kD protein in ring-infected erythrocytes. Perlmann et al. (9) first observed the rim immunofluorescence associated with the erythrocyte membrane of cells infected with young parasites and identified the antigen as a 155-kD protein. This molecule, identified in micronemes of merozoites (12), is presumably inserted into the erythrocyte membrane during invasion. As can be seen in Fig. 1, *c* and *d*, infected mouse erythrocytes have the typical rim-like immunofluorescent pattern of the 155-kD protein.

Although *P. falciparum* merozoites invaded mouse erythrocytes and transformed into ring forms, development did not occur. 36 h after invasion, when late trophozoites and early schizonts had developed in human erythrocytes, no parasites were visible in mouse erythrocytes, suggesting parasite extrusion or lysis of infected erythrocytes.

We injected ring-infected mouse erythrocytes and schizont-infected human erythrocytes into splenectomized B10.BR mice and splenectomized nude (*nu/nu*) mice. After 24 h, no parasites were observed in the peripheral blood of these mice.

Discussion

In this report, we document invasion of mouse and rat erythrocytes by the human malaria parasite *P. falciparum*. Optimal invasion of human erythrocytes by *P. falciparum* merozoites requires sialic acid (1, 2; Hadley, T. J., F. W. Klotz, G. Pasvol, J. D. Haynes, M. H. McGinniss, Y. Okubo, and L. H. Miller, unpublished data); Camp parasites cannot invade in the absence of sialic acid. Although we tested mouse erythrocytes because they contain *N*-acetyl neuraminic acid (3), the same form of sialic acid found on human erythrocytes, additional ligands are probably also involved in invasion. The two-ligand hypothesis was first proposed for *Plasmodium knowlesi* invasion of human erythrocytes (13). In addition to the requirement for multiple ligands for invasion, *P. falciparum* also has heterogeneity among various isolates in its requirements for invasion of human erythrocytes (2; Hadley, T. J., et al., unpublished data). For example, some strains of *P. falciparum* have an almost absolute requirement for sialic acid; others can invade by a sialic acid independent pathway (2; Hadley, T. J., et al., unpublished data). Furthermore, 7G8 parasites invade by a trypsin-sensitive ligand; Camp parasites invade independent of this ligand (Hadley, T. J., et al., unpublished data). This heterogeneity probably explains invasion of rat erythrocytes by the Camp strain of *P. falciparum* but not by the 7G8 strain of *P. falciparum*.

It was assumed that malarias evolved with their hosts, thus the division of malarias into lizard, avian, rodent, and primate malarias (14). From this, one would assume that *P. falciparum*, a human malaria, would not invade rodent erythrocytes unless, by chance, rodent erythrocytes shared common ligands with human erythrocytes. Recently, however, McCutchan et al. (15) grouped *P. falciparum* with avian and rodent malarias and separated *P. falciparum* from the other primate malarias. This classification was dependent on similarities in genome structure. Thus, because of the evolutionary relatedness of *P. falciparum* and rodent malarias, *P. falciparum* merozoites recognize ligands shared by human and mouse erythrocytes. Furthermore, the evolutionary relatedness of avian and rodent malarias may explain the previous observations that the avian parasite, *Plasmodium lophurae*, can multiply in vivo in the erythrocytes of mice and young rats (16) and the rodent malaria, *Plasmodium berghei*, can multiply in duck embryos (17). These considerations may also explain the failure of *P. knowlesi*, a malaria of old world monkeys, to invade erythrocytes of any subprimate (18).

Whatever the implications for merozoite receptor research or parasite evolution, the practical point shown here is the potential use of rodents for vaccine trials using *P. falciparum* antigens from asexual erythrocytic parasites. The limited

supply of primates and the difficulty of trials in humans may slow progress in the development of asexual erythrocytic vaccines. *P. falciparum* adapted to rodents would permit extensive testing for a subunit vaccine, including a mixture of peptides from different parasite proteins. A preliminary study indicated that mice could indeed be infected transiently with *P. falciparum* (19). Now that it has been shown that erythrocyte invasion is not a barrier, it may be possible to adapt *P. falciparum* to growth in vivo in rodents. Because of the inability of *P. falciparum* to develop in mouse erythrocytes after invasion, the development of a rodent model will require further adaptation of the parasite for growth in the mouse erythrocyte or testing of other rodent cells for invasion and growth.

Summary

Plasmodium falciparum malaria merozoites require erythrocyte sialic acid for optimal invasion of human erythrocytes. Since mouse erythrocytes have the form of sialic acid found on human erythrocytes (*N*-acetyl neuraminic acid), mouse erythrocytes were tested for invasion in vitro. The Camp and 7G8 strains of *P. falciparum* invaded mouse erythrocytes at 17–45% of the invasion rate of human erythrocytes. Newly invaded mouse erythrocytes morphologically resembled parasitized human erythrocytes as shown on Giemsa-stained blood films and by electron microscopy. The rim of parasitized mouse erythrocytes contained the *P. falciparum* 155-kD protein, which is on the rim of ring-infected human erythrocytes. Camp but not 7G8 invaded rat erythrocytes, indicating receptor heterogeneity. These data suggest that it may be possible to adapt the asexual erythrocytic stage of *P. falciparum* to rodents. The development of a rodent model of *P. falciparum* malaria could facilitate vaccine development.

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References

1. Hadley, T. J., F. W. Klotz, and L. H. Miller. 1986. Invasion of erythrocytes by malaria parasites: a cellular and molecular overview. *Annu. Rev. Microbiol.* 40:451.
2. Mitchell, G. H., T. J. Hadley, M. H. McGinniss, F. W. Klotz, and L. H. Miller. 1986. Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites: evidence for receptor heterogeneity and two receptors. *Blood.* 67:1519.
3. Reuter, G., J. F. G. Vliegenthart, M. Wember, R. Schauer, and R. J. Howard. 1980. Identification of 9-O-acetyl-*N*-acetylneuraminic acid on the surface of BALB/c mouse erythrocytes. *Biochem. Biophys. Res. Commun.* 94:567.
4. Burkot, T. R., J. L. Williams, and I. Schneider. 1984. Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from the same isolate. *Trans. R. Soc. Trop. Med. Hyg.* 78:339.
5. Trager, W., and J. B. Jensen. 1976. Human malaria parasites in continuous culture. *Science (Wash. DC).* 193:673.
6. Vernes, A., J. D. Haynes, P. Tapcharisri, J. L. Williams, E. Dutoit, and C. J. Diggs.

1984. *Plasmodium falciparum* strain-specific human antibody inhibits merozoite invasion of erythrocytes. *Am. J. Trop. Med. Hyg.* 33:197.

7. Aley, S. B., J. A. Sherwood, and R. J. Howard. 1984. Knob-positive and knob-negative *Plasmodium falciparum* differ in expression of a strain-specific malarial antigen on the surface of infected erythrocytes. *J. Exp. Med.* 160:1585.
8. Hadley, T. J., Z. Erkmen, B. M. Kaufman, S. Futrovsky, M. H. McGinniss, P. Graves, J. C. Sadoff, and L. H. Miller. 1986. Factors influencing invasion of erythrocytes by *Plasmodium falciparum* parasites. *Am. J. Trop. Med. Hyg.* 35:898.
9. Perlmann, H., K. Berzins, M. Wahlgren, J. Carlsson, A. Bjorkman, M. E. Patarroyo, and P. Perlmann. 1984. Antibodies in malarial sera to parasite antigens in the membrane of erythrocytes infected with early asexual stages of *Plasmodium falciparum. J. Exp. Med.* 159:1686.
10. Johnson, G. D., and G. M. de C. Nogueira Araujo. 1981. A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods.* 43:349.
11. Haynes, J. D., C. L. Diggs, F. A. Hines, and R. E. Desjardins. 1976. Culture of human malaria parasites *Plasmodium falciparum. Nature (Lond.).* 263:767.
12. Brown, G. V., J. G. Culvenor, P. E. Crewther, A. E. Bianco, R. L. Coppel, R. B. Saint, H-D. Stahl, D. J. Kemp, and R. F. Anders. 1985. Localization of the ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* in merozoites and ring-infected erythrocytes. *J. Exp. Med.* 162:774.
13. Miller, L. H., M. Aikawa, J. G. Johnson, and T. Shiroishi. 1979. Interaction between cytochalasin β-treated malarial parasites and erythrocytes. Attachment and junction formation. *J. Exp. Med.* 149:172.
14. Garnham, P. C. C. 1966. Classification and evolution. *In* Malaria Parasites. Blackwell Scientific Publications, Oxford. 60–80.
15. McCutchan, T. F., J. B. Dame, L. H. Miller, and J. Barnwell. 1984. Evolutionary relatedness of *Plasmodium* species as determined by the structure of DNA. *Science (Wash. DC).* 225:808.
16. McGhee, R. B. 1953. The influence of age of the animal upon the susceptibility of mammalian erythrocytes to infection by the avain malaria parasite *Plasmodium lophurae. J. Infect. Dis.* 92:4.
17. McGhee, R. B. 1954. The infection of duck and goose erythrocytes by the mammalian malaria parasite: *Plasmodium berghei. J. Protozool.* 1:145.
18. Mason, S. J., L. H. Miller, T. Shiroishi, J. A. Dvorak, and M. H. McGinniss. 1977. The Duffy blood group determinants: their role in the susceptibility of human and animal erythrocytes to *Plasmodium knowlesi* malaria. *Br. J. Haematol.* 36:327.
19. Weinman, D., D. C. Cavanaugh, and R. S. Desowitz. 1966. *Plasmodium falciparum* in mus musculus. *Trans. R. Soc. Trop. Med. Hyg.* 60:562.